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# Molecular Cloning and Biological Characterization of Full-Length HIV-1 Subtype C from Botswana

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Human immunodeficiency virus type 1 (HIV-1) subtype C is now responsible for more than half of all HIV-1 infections in the global epidemic and for the high levels of HIV-1 prevalence in southern Africa. To facilitate studies of the biological nature and the underlying molecular determinants of this virus, we constructed eight full-length proviral clones from two asymptomatic and three AIDS patients infected with HIV-1 subtype C from Botswana. Analysis of viral lysates showed that Gag, Pol, and Env structural proteins were present in the virions. In four clones, the analysis suggested inefficient envelope glycoprotein processing. Nucleotide sequence analysis of the eight clones did not reveal frameshifts, deletions, premature truncations, or translational stop codons in any structural, regulatory, or accessory genes. None of the subtype C clones were replication competent in donor peripheral blood mononuclear cells (PBMCs), macrophages, Jurkat$_{tat}$ cells, or U87.CD4.CCR5 cells. However, infection by two clones could be rescued by complementation with a functional subtype C envelope clone, resulting in a productive infection of PBMCs, macrophages, and U87.CD4.CCR5 cells. 

## INTRODUCTION

The human immunodeficiency virus type 1 (HIV-1) pandemic is characterized by extensive genetic variation as a result of a high error rate of the viral reverse transcriptase (RT) and RNA polymerase, high viral replication, viral genome recombination, immune selection, and viral phenotype switching (Drosopoulos *et al.,* 1998; Preston *et al.,* 1988). HIV-1 sequences have been classified into three groups, designated M (major), O (outlier), and N (non-M, non-O) (Gurtler *et al.,* 1994; Kostrikis *et al.,* 1995; Leitner *et al.,* 1995; Loussert-Ajaka *et al.,* 1995; Myers *et al.,* 1995; Simon *et al.,* 1998; Vanden Haesevelde *et al.,* 1994). The majority of HIV-1 infections are caused by viruses in the M group, which is subdivided into at least 10 genetic subtypes. The global distribution of the subtypes is uneven, with subtypes A, C, and D predominating in sub-Saharan Africa, subtype B in Europe, North America, and South America, and subtypes C and E in Asia. Subtype C is the dominant subtype in regions with the highest prevalence of HIV-1 infection, including South Africa, Malawi, Zambia, Namibia, Zimbabwe, Ethiopia, and Botswana (Birx *et al.,* 1996; Essex, 1999; Hu *et al.,* 1996; Ping *et al.,* 1999). Subtype C is also the predominant subtype in India (Cassol *et al.,* 1996; Dietrich *et al.,* 1993; Galai *et al.,* 1997; Hu *et al.,* 1996).

Most information on the molecular mechanisms underlying HIV-1 pathogenesis has come from primary isolates and subtype B clones. Recently, near full-length genome non-B subtype viral sequences have been reported (Gao *et al.,* 1998; Lole *et al.,* 1999; Novitsky *et al.,* 1999). Biological and phenotypic characterizations of HIV-1 subtype C molecular clones have not been reported. Amino acid sequence analysis shows that subtype differences reach 15, 10, and 24% in Gag, Pol, and Env, respectively, raising the possibility of biological differences between subtypes (Gao *et al.,* 1998).

Viral molecular clones can be used to correlate genotype to phenotype by the introduction of specific mutations or other genomic alterations. Viral clones offer the advantage of studying a virus as a unit rather than as different subgenomic fragments. In this report, we describe the generation of eight HIV-1 subtype C full-length molecular clones from five different individuals. We analyzed the protein profile of viral supernatants and their ability to infect primary and continuous cell lines. A Botswana HIV-1 subtype C gp160 clone was able to complement *in trans* and render two clones infectious to peripheral blood mononuclear cells (PBMCs), macrophages, and U87.CD4.CCR5 cells, although individual noncomplemented virus stocks from transfected cells were unable to establish a productive infection of target cells.

## RESULTS

### PCR amplification and cloning

Using the approaches outlined in Fig. 1, we successfully amplified and cloned eight full-length clones from

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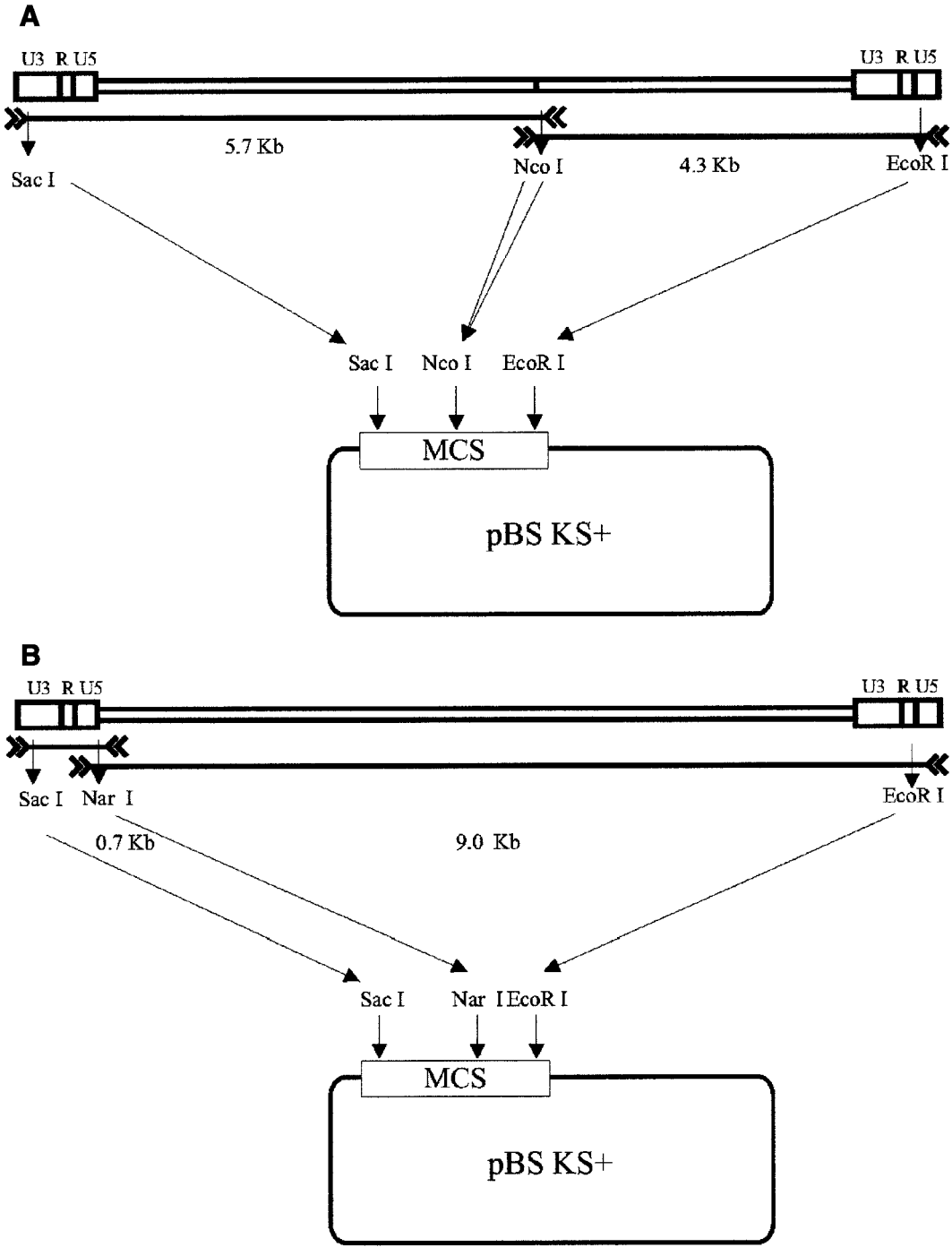


**FIG. 1.** PCR amplification and cloning strategies. (A) Two subgenomic fragments were amplified. The 5′ fragment was amplified using a sense primer with a *Sac*I restriction site while the antisense primer had an *Eco*RI site. The 3′ fragment was amplified using a sense primer with an *Nco*I site and the antisense primer had an *Eco*RI site. The 5′ fragment was cloned into the *Sac*I and *Eco*RI sites of pBluescript KS(+) (pBS-KS+) plasmid. The 3′ fragment was then subcloned by digesting this plasmid with *Nco*I (partial digest) and *Eco*RI. (B) 5′ LTR was amplified and cloned into the multiple cloning site of pBS-KS+ (*Sac*I and *Eco*RI sites). A 9-kb HIV-1C fragment previously amplified from genomic DNA and cloned into pCR 2.1 vector was then digested and cloned into the *Nar*I (in 5′ LTR) and the *Eco*RI sites (in vector) to restore the full proviral genome.

five HIV-1 seropositive patients. All clones were confirmed to contain HIV-1 sequences by Southern blot analysis using probes corresponding to the LTR, *gag,* and *env* genes. All clones represent primary isolates since they were obtained directly from patients' blood or propagated for 14 days in PBMCs.

### Generation of virus stocks

Molecular clones were transfected into 293T, COS-1, and COS-7 cells to examine their ability to generate infectious virus stocks. These three cell lines were used because they are known to efficiently produce infectious HIV-1 virions upon transfection but are not susceptible to infection by free virions because they lack the receptors needed for viral entry into cells. Production of viral particles was assessed by quantification of p24 antigen and RT in tissue culture supernatants. For all three cell lines, the clones released large amounts of p24 antigen and RT by 60 h posttransfection. All three cell lines showed a similar viral protein profile from their culture supernatants as assessed by Western blot (data not shown). As expected there was correlation between the amounts of p24 antigen and RT activity in each culture supernatant. When these supernatants were used to infect donor PBMCs, macrophages, U87.CD4.CCR5 cells, or Jurkat$_{tat}$ cells, none of the viral supernatants was able to establish a productive infection as determined by the failure to observe an increase in p24 antigen in the cell supernatants over a 21-day period (not shown).

### Western blot analysis

In an attempt to identify possible reasons for the lack of infectivity of these molecular clones, we carried out Western blot analysis of viral pellets from supernatants of transfected cells. Four clones did not express the entire range of virion-associated proteins known to be necessary for productive infection. Four clones, designated C.96BW06.H51 (H51), C.96BW06.J4 (J4), C.96BW06.J7 (J7), and C.96BW06.K18 (K18), appeared to express all virion-associated proteins as did the subtype B molecular clone HXB2RU3CI (Fig. 2). We therefore decided to concentrate further study on these four clones. Results from several blots suggested that although these four clones expressed all the expected virion-associated proteins, they showed large amounts of unprocessed gp160 polyprotein relative to processed gp120. It was previously shown that impairment in the processing of the gp160 envelope precursor can result in loss of viral infectivity (Dedera *et al.,* 1992; Guo *et al.,* 1990; Syu *et al.,* 1991). Our results thus led us to speculate that inefficient processing of the envelope protein coupled with packaging of this nonfunctional form of the protein into virions might explain the lack of infectivity of these clones.

### Envelope complementation of replication-defective clones

To test whether these four clones were packaging an unprocessed or defective envelope protein that resulted in a lack of infectivity, we took advantage of a previously described complementation assay that identifies functional envelope proteins (Helseth *et al.,* 1990). Using this

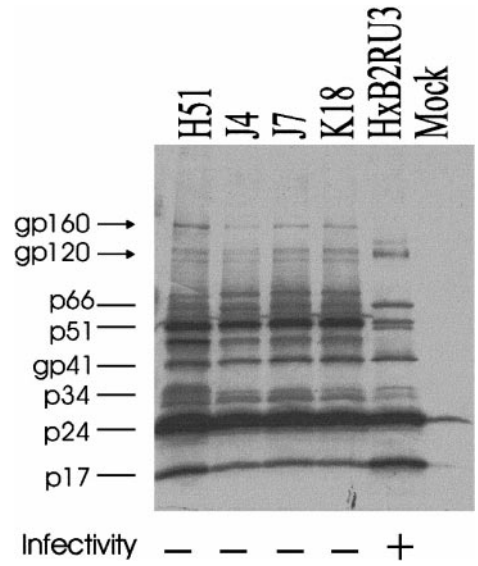


FIG. 2. Analysis of viral proteins by Western blot. COS-7, 293T, and COS-1 cells were transfected with plasmid DNA containing the full-length genomes of HIV-1 proviruses. Cell culture supernatants were collected at 72 h posttransfection and overlaid on a 20% sucrose cushion. After centrifugation, viral pellets were resuspended in lysis buffer and viral proteins were separated by 4–15% linear SDS–PAGE. Proteins were transferred to nitrocellulose membranes and analyzed by immunoblot with human sera from an individual from Botswana infected with HIV-1 subtype C.

system (with the modifications outlined under Materials and Methods), we identified a functional HIV-1 subtype C envelope clone from Botswana designated pSVIII/MOLE1 (data not shown). All nine of the other envelope clones repeatedly yielded chloramphenical acetyl transferase (CAT) levels lower than those obtained for the positive control 89.6 envelope and were thus discarded. When the pSVIII/MOLE1 clone was cotransfected with each of the four full-length clones and the supernatants used to infect PBMCs, macrophages, and U87.CD4.CCR5 cells, we found that in all cases, clones C.96BW06.J4 and C.96BW06.J7 became infectious while clones C.96BW06.H51 and C.96BW06.K18 remained noninfectious (Fig. 3). In all three cell types, 500 pg total p24 antigen was used to infect cells. The amount of p24 antigen in the tissue culture supernatant of PBMCs infected with C.96BW06.J4 (+MOLE1 env) increased steadily and appeared to peak by day 15 (Fig. 3A). P24 amounts increased slowly for C.96BW06.J7 (+MOLE1 env) infection of PBMCs but they were still rising by day 21 postinfection. The titers of p24 antigen in all subtype C clones were lower than for the subtype B clone, HXB2RU3CI. In macrophages, all three clones appeared to peak in p24 production by day 15, with virus production for C.96BW06.J7 (+MOLE1 env) being about 50% of what was observed for C.96BW06.J4 (+MOLE1 env) and HXB2RU3CI (Fig. 3B). In U87.CD4.CCR5 cells, virus production showed a sharp increase during the first week of infection for all clones. Peak p24 production for the

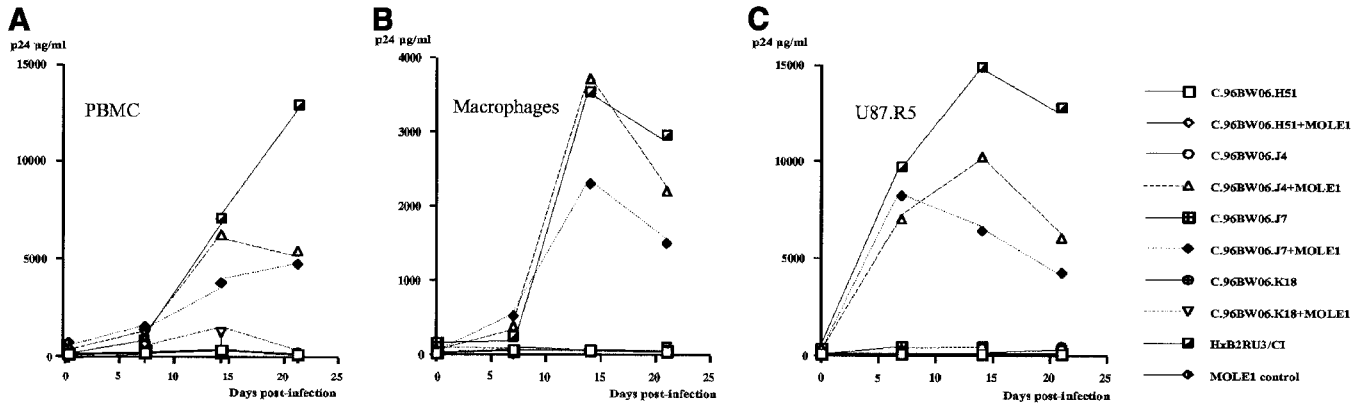


FIG. 3. Complementation of the full-length clones with a functional envelope (MOLE1) renders them infectious in (A) PBMCs, (B) macrophages, and (C) U87.CD4.CCR5 cells. COS-1 cells were cotransfected with 2 and 1 $\mu$g of full-length and pSVIII/MOLE1 envelope clone, respectively. 72 h later, p24 antigen was quantified by ELISA. Culture supernatant corresponding to 500 pg of p24 was then used to infect $3 \times 10^6$ cells. The following day, the target cells were washed three times with PBS and then new medium was added. Infection was monitored by p24 ELISA.

subtype C clones was about 65% of the HXB2RU3CI subtype B positive control. In all three cell types, none of the subtype C full-length clones replicated without the pSVIII/MOLE1 envelope clone complementation. As expected, transfection of and infection by pSVIII/MOLE1 construct alone did not result in viral production.

Western blot analysis suggested inefficient gp160 processing as a possible mechanism for the lack of infectivity of the full-length clones. We therefore analyzed the effect of the pSVIII/MOLE1 envelope construct cotransfection on the Western blots of clones C.96BW06.H51, C.96BW06.J4, and C.96BW06.J7 (Fig. 4). Amounts of gp120 relative to gp160 were analyzed using NIH Image software. We observed that pSVIII/MOLE1 complementation of C.96BW06.J4 and C.96BW06.J7 clones resulted in a gp120 to gp160 ratio of approximately 2 (1.9), whereas for C.96BW06.H51, the ratio was about 1. The ratio in the noncomplemented noninfectious clones was approximately 1 in all cases. This result suggests a deficient processing of gp160 and that a certain threshold ratio of virion-packaged gp120 to gp160 may be necessary for these subtype C clones to be infectious.

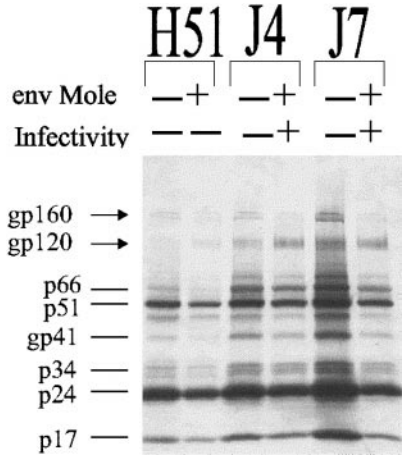


FIG. 4. Western blot analysis of full-length viral lysates with and without complementation with MOLE 1 envelope. Serum from an individual infected with HIV-1 subtype C was used to identify the proteins.

## Sequence analysis

Nucleotide sequencing of the entire genome was carried out for the four clones that expressed all virion-associated proteins to confirm that all HIV-1 structural, regulatory, and accessory genes were preserved. We did not identify any obvious inactivating mutations such as translational stop codons, frameshifts, premature truncations, or deletions in *gag, pol, env, nef, rev, tat, vif, vpr,* or *vpu.* The LTR sequences were intact with motifs such as the three NF-$\kappa$B binding sites, the Sp1 binding site, and the primer binding site preserved. The RNA packaging signal sequence was present in all the clones. Phylogenetic analysis and variability plots confirmed the clones to be of subtype C in every region of the genome (not shown). An examination of the inferred V3 loop amino acid sequences (Fig. 5) led us to predict that the new sequences would most likely be of the NSI/R5 phenotype as evidenced by a lack of basic amino acids at positions 11 and 25. We therefore decided to compare the new V3 sequences with those from a known CCR5-using subtype B clone, B.US.YU2. (Li *et al.,* 1991; Choe *et al.,* 1996, 58). There were seven amino acid differences in the V3 between the new sequences and the B.US.YU2 molecular clone. Most of the clones differed from the consensus subtype C sequence by an isoleucine substitution for valine at position 12. C.96BW06.K18 and C.96BWMOLE1 maintained the valine residue while C.96BW0407 had a methionine substitution at this position. C.96BW06.J4, C.96BW06.J7, and 96BW01B22 had a histidine to tyrosine substitution at position 34. Clones C.96BW06.J4 and C.96BW06.J7 had single amino acid deletions of glutamine 24 in the V3 loop.

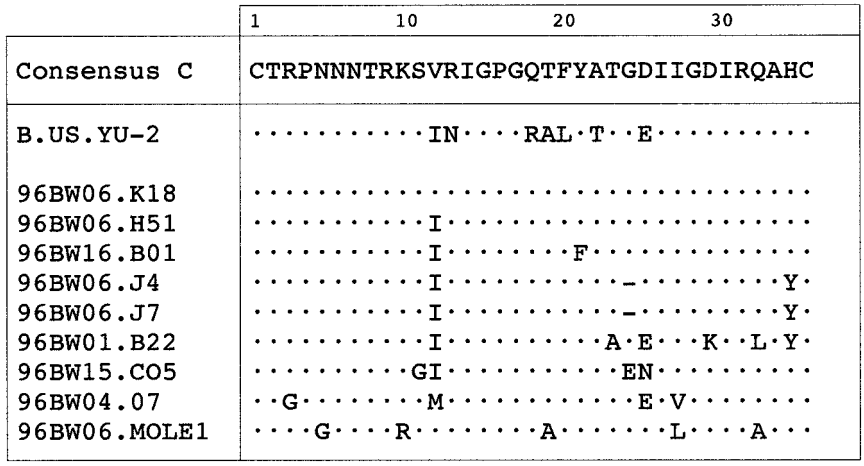


FIG. 5. Envelope V3 alignment of the eight sequenced clones. The sequences are compared to the consensus subtype V3. The consensus was generated using the subtype C genomes from the HIV-1 database (Korber *et al.,* 1997).

Since we observed in the Western blot that the processing of gp160 to gp120 and gp41 was deficient, we investigated whether the known proteolytic cleavage site was conserved. The site sequence REKR was conserved in all the envelopes except in C96BW06.H51 where the second arginine is replaced by serine. Interestingly, it has previously been shown that an arginine to threonine substitution at that position affects gp160 envelope proteolytic cleavage in subtype B viruses (Guo *et al.,* 1990).

## DISCUSSION

In this study, we have cloned, sequenced, and biologically characterized eight full-length HIV-1 subtype C viruses from Botswana. Our main findings were that two clones (C96BW06.J4 and C.96BW06.J7) generated infectious viral stocks when complemented with an exogenous envelope protein from another subtype C sample from Botswana. None of the virus stocks obtained after individual transfection of the eight molecular clones (C.BW01.B16, C.BW04.07, C.96BW06.H51, C96BW06.J4, C.96BW06.J7, C.96.BW06.K18, C.96BW15.C2, and C.96BW16.26) were infectious when tested in PBMCs, macrophages, Jurkat$_{tat}$ cells, and U87.CD4.CCR5 cells. We envision that these clones will allow us to study in detail the effects of molecular changes on the ability of subtype C viruses to infect cells, replicate, and cause cytopathic effects. To our knowledge, this is the first report in which subtype C infectious complements have been obtained directly from infected PBMCs. It has previously been shown that clones obtained after *in vitro* passage may have altered biological characteristics that may render them somewhat less suitable in studies of mechanisms underlying pathogenesis (Sullivan *et al.,* 1995). Our infection system, while still limited by the need to carry out complementation, may overcome some of these problems.

We suggest at least two potential mechanisms (or their combination) of the observed HIV-1 infection seen upon transfection with the envelope clone. The first might be a simple complementation that leads to only one round of infection since the genome packaged into virions is defective. The second mechanism might involve a recombination event resulting in repair of the defective genome, as has been previously observed (Sadaie *et al.,* 1992). Further study is warranted to evaluate the contribution of each component/mechanism to the HIV-1 subtype C infection seen. Attempts are also ongoing to construct a full-length clone from the sample C96BW.MOLE1 in order to further characterize this virus. An infectious subtype C molecular clone has previously been reported from an Indian sample after *in vitro* culture of virus in continous cell lines (Mochizuki *et al.,* 1999). The clones described in this article were obtained directly from PBMCs of infected persons or were cultured only briefly in donor PBMCs and therefore represent primary isolates. Importantly, samples from which these subtype C clones were generated are from southern Africa, where some of the highest HIV-1 seroprevalence rates in the world are found.

There are previous reports that defective viral genomes are frequently encountered in the PBMCs of HIV-1-infected individuals (Sanchez *et al.,* 1997). In such cases, it has been possible to define the molecular basis for replication incompetence. It is puzzling that no obvious mutations could account for lack of infectivity in all the sequenced clones in our study. Our results suggest that beyond obvious inactivating mutations, the molecular determinants of viral infectivity may be more complex than previously thought.

Subtype C viruses display certain unique genetic characteristics that may lead to altered biological activity. These viruses possess Tat and Rev proteins that are prematurely truncated and they have a 15-bp insertion at the 5′ end of the *vpu* reading frame (Gao *et al.,* 1998). So far, no unique biological characteristics have been

TABLE 1

Demographic and Clinical Characteristics of Patients and Samples

| Patient | Age | Sex | City of origin in Botswana | CDC stage | No. of full-length clones |
|---|---|---|---|---|---|
| 96BW01 | 29 | M | Gaborone | II | 1 |
| 96BW04 | 43 | M | Gaborone | II | 1 |
| 96BW06 | 25 | M | Gaborone | I | 4 |
| 96BW15 | 22 | F | Gaborone | IVC | 1 |
| 96BW16 | 28 | F | Gaborone | IVC | 1 |
| 96MOLE1 | 23 | M | Molepolole | III | 1[a] |

[a] The functional envelope clone was amplified from this sample.

mapped to these genetic features. Recently, it has been shown that subtype C viruses almost exclusively use the CCR5 chemokine receptor as a coreceptor for entry into cells while other subtypes employ other coreceptors as well (Bjorndal *et al.,* 1999; Ping *et al.,* 1999; Tscherning *et al.,* 1998). Since this coreceptor appears to be overrepresented in the female genital tract (Patterson *et al.,* 1998), it is plausible that subtype C viruses are more efficient in infecting the cells that line the genital mucosa. We and others have also shown that HIV-1C viruses possess three or four NF-$\kappa$B enhancer copies in the LTR compared to the conventional two copies in subtype B (Gao *et al.,* 1996; Montano *et al.,* 1997; Salminen *et al.,* 1996). We have further demonstrated that the extra NF-$\kappa$B sites in HIV-1 subtype C are associated with increased responsiveness to p65/Rel A and to the proinflammatory cytokine TNF-$\alpha$ (Montano *et al.,* 1997, 2000). Others have shown that HIV-1 subtypes may differ in the promoter/enhancer activities of their LTRs, with HIV-1C displaying higher activity levels (Naghavi *et al.,* 1999).

There is some evidence that infection with HIV-1 subtype C may be associated with higher plasma RNA levels and significantly lower CD4 lymphocyte counts than subtypes A or D (Neilson *et al.,* 1999). It has also been suggested that HIV-1 genotypes may be spreading at different rates in Tanzania, with subtype C viruses being at an advantage (Renjifo *et al.,* 1998). In Senegal, subtype C appeared to be associated with faster disease progression than subtype A (Kanki *et al.,* 1999). Recombinant genomes of HIV-1 in Tanzania have also been found to contain an uneven representation of the envelope V3 loop from different subtypes, with subtype C V3 having a higher representation than would be expected (Renjifo *et al.,* 1999). Although these studies are invaluable in tracking changes in the HIV-1 epidemic landscape, they are unable to provide biological explanations for the observed trends. Studies with HIV-1 molecular clones such as those described in this report may help provide some answers to these questions. Finally, the reagents described in this report are expected to be useful as a source of antigens and in immunological response assays in the continuing effort to develop a clade C vaccine for the southern Africa region.

## MATERIALS AND METHODS

### Samples and DNA extraction

Samples for full-length HIV-1 amplification and cloning were obtained from anonymous donors in Gaborone and Molepolole, Botswana (Table 1). Patients' serostatus was established by enzyme-linked immunosorbent assay (ELISA) and Western blot analysis. Information regarding time and route of infection is not known. Patient clinical classification was carried out according to the Centers for Disease Control and Prevention revised criteria (CDC, 1987).

Genomic DNA was extracted directly from patients' PBMCs using the QIAamp Blood kit (Qiagen, Chatsworth, CA). For samples 96BW06 and 96MOLE1, patients' PBMCs were cocultured in donor PBMCs for 2 weeks prior to DNA extraction.

### PCR amplification and plasmid constructs

We used the Expand Long Template PCR System to amplify the viral genome (Boehringer Mannheim, Indianapolis, IN). Two approaches were employed to clone and construct full-length HIV-1 proviral genomes (Fig. 1). In the first strategy, which was used for sample 96BW06 (four clones), the viral genome was amplified in two pieces, a 5.7-kb (5′ LTR to *vif*) fragment and a 4.3-kb (*vif* to 3′ LTR) fragment (Fig. 1A). The primers used for the amplification of the 5′ fragment (5.7 kb) were U3-SAC (5′-CCGCGGGCGAGCTCGTGGAAGGGTTAATTTACTCTAAGAAAAGGC-3′) and BG15-NR (5′-CGCGGATCCCCATGGTGGAGGAAAGTGTCTGACAGCTTCCTG-3′). Primers for amplification of the 3′ fragment (4.3 kb) were BG15-NF (5′-CGCGGATCCGACACTTTCCTCCACCATGGCTCCATAGCTTAG-3′) and U5-R (5′-CGCGGATCCGCGGCGGCCGCGCACCCATCTCTCTCCTTCTAGCCT-3′). PCR products were first cloned into the pCR 2.1-Topo vector (InVitrogen, San Diego, CA) and propagated in JM109 competent cells (Promega Corp., Madison, WI).

The 5′ fragment was then digested with *Sac*I and *Eco*RI restriction enzymes and ligated into a pBluescript KS(+) (pBS-KS+) vector (Stratagene, La Jolla, CA) that had been linearized using the same enzymes. The 3′ fragment was then subcloned into this pBS-KS+ vector by digestion with *Nco*I (partial digestion) and *Eco*RI enzymes.

The second strategy was to reconstruct four near-full-length sequences, previously submitted to GenBank (Accession Nos. AF110961, AF110963, AF11075, and AF110976), by insertion of the 5′ LTR (Accession No. AF290031) (Fig. 1B). A 9-kb fragment was amplified using a heminested approach with the LA set of primers (Fujii *et al.,* 1997). In the first round amplification, 1 μg of DNA was used as a template with primers LA1 and LA2. A total volume of 50 μl per PCR was used with a 300 μM concentration of each primer. We used 1.75 mM $MgCl_2$ with 350 μM dNTPs per reaction. The PCR product was run on 0.9% agarose gel and purified using the Qiaex II gel extraction kit (Qiagen). Primers LA3 and LA2 were used for the second-round reaction, with 5 μl of purified PCR product from the first-round reaction. Cycling conditions for both rounds of the PCR were as recommended by the manufacturer. After initial cloning of the PCR product in pCR 2.1 Topo vector, this fragment was subcloned into a pB-KS+ vector into which a subtype C LTR had previously been cloned in the *Sac*I and *Eco*RI sites. Primers used for amplification of the 5′ LTR were U3-SAC and PBS-ER (5′-CGCGTCGACCCGGAATTC-CTCTCCTTCTAGCCTCCGCTAGTC-3′). The sites used for the subcloning were *Nar*I, which was found to occur naturally near the primer binding site in most LTR sequences, and *Eco*RI in the pBS-KS+ vector sequence. Recombinant clones were screened by restriction analysis, PCR, and Southern blot using LTR, *gag,* and *env* primers (data not shown). Clones with full-length genomes were used for maxiprep DNA.

## Envelope expressor plasmid

Genomic DNA was subjected to PCR amplification using the Expand Long Template PCR system with the following cycling conditions: a 2-min denaturation at 94°C followed by 10 cycles of 10 s at 94°C, 30 s at 57°C, 3 min at 68°C, and then another 20 cycles under these conditions but with an additional 15 s per cycle for each 68°C extension step. The final extension was at 68°C for 7 min. The primers used were ETH-KPN (5′-TATGGGG-TACCTGTGTGG-3′) and ETH-BAM (5′-CTAAGGATCCGT-TCACTAATC-3′). To create the MOLE 1 envelope expressor plasmid (pSVIII/MOLE1), the *Kpn*I (6347)–*Bam*HI (8475) fragment of the pSVIIIenv plasmid (Helseth *et al.,* 1990) was replaced with the PCR amplicon from the MOLE1 sample. Nine other subype C envelope clones—each from a different individual—were likewise constructed for functional analysis. The envelope clones were then sequenced.

## Cells and cell lines

293T cells were propagated in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). COS-1 and COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. U87.CD4.CCR5 cells were maintained in DMEM supplemented with 15% FBS, 1 μg/ml puromycin, and 300 μg/ml G418. $Jurkat_{tat}$ cells were maintained in RPMI 1640 with 10% FBS and 800 μg/ml G418. PBMCs and macrophages were obtained from HIV-1 seronegative anonymous healthy donors and separated by density-gradient centrifugation on lymphocyte separation medium (Organon Teknika, Corp., Durham, NC). PBMCs were cultured in RPMI 1640 supplemented with 10% FBS and were stimulated with 5 μg/ml phytohemagglutinin (Sigma, St. Louis, MO) and 20 U/ml of interleukin-2 (Becton Dickinson Labware, Bedford, MA) for 48 to 72 h prior to infection.

## Transfection and infection

Subconfluent COS-1, COS-7, and 293T cells were transfected with 2–5 μg of plasmid DNA using the Fugene 6 transfection reagent (Boehringer Mannheim). HXB2RU3CI, a macrophage-tropic subtype B molecular clone (Trujillo *et al.,* 1996; Wang *et al.,* 1998), was used as the positive control. Sixty to 72 h posttransfection, culture supernatants were filtered through 0.2-μm-pore-size filter units (Nalgene, Rochester, NY) and HIV-1 virions were quantified by p24 antigen ELISA (NEN Life Science Products, Boston, MA) and by the *Quan*-T-RT [$^3$H] reverse transcriptase assay (Amersham Pharmacia Biotech, Piscataway, NJ). Equivalent amounts of virus (as determined by amount of p24 antigen in culture supernatant) were used to infect $3 \times 10^6$ PBMCs, macrophages, U87.CD4.CCR5 glioma cells, and $Jurkat_{tat}$ cells. Twenty-four hours postinfection, cells were washed three times with phosphate-buffered saline and fresh medium was then added. Infection of the target cells was monitored for up to 21 days by p24 ELISA.

## Envelope function analysis

To examine the ability of the subtype C envelope clones to complement cell-free viral transmission, we followed the method previously described by Helseth *et al.* (1990) with some modifications. Briefly, COS-1 cells were cotransfected with 1 μg pHXBΔenvCAT and 2 μg of the envelope expressor plasmid. The dual-tropic 89.6 envelope expressor plasmid (Sullivan *et al.,* 1995) was used as a positive control. At 72 h posttransfection, the cell supernatants were filtered through 0.2-μm-pore-size filter units (Nalgene) and p24 antigen was quantified by ELISA (NEN Life Science Products). We then used su-

pernatant containing 500 pg of p24 antigen to infect $5 \times 10^5$ U87.CD4.CCR5 cells in 2 ml of medium. The cells were washed three times the following day with PBS. Sixty to 72 h later, all supernatant was removed, and the cells were washed three times with PBS. The cells were then lysed and the amount of CAT in the lysate was determined using the CAT ELISA kit (Boehringer Mannheim). A total of 10 different subtype C envelope clones were examined using this protocol.

### Viral pellets

To prepare viral proteins for Western blot analysis, filtered culture supernatants from transfected cells were centrifuged at 3000 rpm for 15 min. Supernatants were then overlaid on 4 ml of 20% sucrose cushion and centrifuged at 20,000 rpm (Beckman SW28 rotor) for 2 h. The supernatant was then discarded and any remaining fluid was dried using cotton swabs. The viral pellet was then resuspended in 100–150 $\mu$l of lysis buffer (0.15 M NaCl, 0.05 M Tris–HCl, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate). The total amount of protein recovered was quantified by the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA). Twelve micrograms of total protein for each sample was then mixed with reducing buffer (0.08 M Tris–HCl, pH 6.8, 0.1 M dithiothreitol, 2% sodium dodecyl sulfate, 10% glycerol, and 0.2% bromophenol blue), boiled for 3 min, and resolved by 4–15% linear sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

### Immunoblotting

Resolved proteins were transferred passively by placing the gel between two nitrocellulose membranes (Schleicher and Schuell, Keene, NH) and placing a weight on top of the cassette for 48 h. Viral proteins were visualized by immunoblotting with a range of sera from HIV-1 seropositive individuals infected with subtype C from Botswana and individuals infected with subtype B from North America. Analysis of amounts of gp120 protein relative to gp160 in the viral lysate was performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

### Sequencing and sequence analysis

A primer walking strategy on purified plasmid DNA was used for sequencing both strands of the genome, with overlapping contiguous sequences obtained throughout the genome to ensure accuracy of sequence output (primers were approximately 300 bp apart on each strand of the genome). Over 100 different sequencing primers were used, and their sequences are available on request. Automatic sequencing was carried out using a Model 373A automated DNA Sequenator (Applied Biosystems, Inc., Foster City, CA). Individual contiguous sequences of proviral DNA were assembled using the Sequencher program (Gene Codes Corp., Ann Arbor, MI). Multiple sequence alignment was carried out using the clustal W program and the Lasergene program. The Njplot (Perriere and Gouy, 1996) and Treeview (Page, 1996) programs were used to view sequence relatedness. The program SWAN (Proutski and Holmes, 1998), using a 300-bp sliding window with a step-wise increment of 30 bp, assessed variability patterns. All gaps were excluded during sequence analysis. The Recombinant Identification Program (Siepel *et al.,* 1995) and HIV Subtyping Basic BLAST (Altschul *et al.,* 1997) were used to assess viral recombination (data not shown).

### GenBank accession numbers

New sequences described in this study are available under GenBank Accession Nos. AF290027–AF290032.

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## REFERENCES

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipmann, D. J. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acid Res.* **25,** 3389–3402.

Birx, D., VanCott, T., Michael, N., McNeil, J., Stamatos, N., Gilliam, B., Davis, R., Carr, J., and McCutchan, F. (1996). Summary of track A: Basic science. *AIDS* **10,** S85–S106.

Bjorndal, A., Sonnerborg, A., Tscherning, C., Albert, J., and Fenyo, E. (1999). Phenotypic characteristics of human immunodeficiency virus type 1 subtype C isolates of Ethiopian AIDS patients. *AIDS Res. Hum. Retroviruses* **15**(7), 647–653.

Cassol, S., Weniger, B., Babu, P., Salminen, M., Zheng, X., Htoon, M., Delaney, A., O'Shaughnessy, M., and Ou, C. (1996). Detection of HIV type 1 env subtypes A, B, C, and E in Asia using dried blood spots: A new surveillance tool for molecular epidemiology. *AIDS Res. Hum. Retroviruses* **12,** 1435–1441.

Centers for Disease Control and Prevention (CDC) (1987). Revision of the CDC surveillance case defination for acquired immunodeficiency syndrome. *Morbidity Mortality Weekly R. P.* **36,** Suppl. 1 S1–S15.

Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Ponath, P. D., Wu, L., Mackay, C. R., LaRosa, G., Newman, W., Gerard, N., Gerard, C., and Sodroski, J. (1996). The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* **85,** 1135–1148.

Dedera, D., Gu, R., and Ratner, L. (1992). Conserved cysteine residues in the human immunodeficiency virus type 1 transmembrane envelope protein are essential for precursor envelope cleavage. *J. Virol.* **66**(2), 1207–1209.

Dietrich, U., Grez, M., von Briesen, H., Panhans, B., Geissendorfer, M., Kuhnel, H., Maniar, J., Mahambre, G., Becker, W. B., Becker, M. L. B., and Rubsamen-Waigmann, H. (1993). HIV-1 strains from India are highly divergent from prototypic African and US/European strains, but are linked to a South African isolate. *AIDS* **7**(1), 23–27.

Drosopoulos, W., Rezende, L., Wainberg, M., and Prasad, V. (1998).

Virtues of being faithful: Can we limit the genetic variation in human immunodeficiency virus? *J. Mol. Med.* **76**(9), 604–612.

Essex, M. (1999). Human immunodeficiency viruses of the developing world. *Adv. Virus Res.* **53,** 71–88.

Fujii, S., Obaru, K., Matsushita, S., Morikita, T., Higuchi, H., Fujimoto, K., and Takatsuki, K. (1997). Characterization of proviral DNA from an individual with long-term, non-progressive infection with HIV-1 and non-recoverable virus. *J. Acquired Immune Defic. Syndr. Hum. Retrovirol.* **15,** 247–256.

Galai, N., Kalinkovich, A., Burstein, R., Vlahov, D., and Bentwich, Z. (1997). African HIV-1 subtype C and rate of progression among Ethiopian immigrants in Israel. *Lancet* **349,** 180–181.

Gao, F., Robertson, D., Carruthers, C., Morrison, S., Jian, B., Chen, Y., Barre-Sinoussi, F., Girard, M., Srinivasan, A., Abimiku, A., Shaw, G., Sharp, P., and Hahn, B. (1998). A comprehensive panel of near-full-length clones and reference sequences for non-subtype B isolates of human immunodeficiency virus type 1. *J. Virol.* **72**(7), 5680–5698.

Gao, F., Robertson, D., Morrison, S., Hui, H., Craig, S., Decker, J., Fultz, P., Girard, M., Shaw, G., Hahn, B., and Sharp, P. (1996). The heterosexual human immunodeficiency virus type 1 epidemic in Thailand is caused by an intersubtype (A/E) recombinant of African origin. *J. Virol.* **70,** 7013–7029.

Guo, H., Veronese, F., Tschachler, E., Pal, R., Kalyanaraman, V., Gallo, R., and Reitz, M. J. (1990). Characterization of an HIV-1 point mutant blocked in envelope glycoprotein cleavage. *Virology* **174**(1), 217–224.

Gurtler, L., Hauser, P., Erberle, J., von Brunn, A., Knapp, S., Zekeng, L., Tsague, J., and Kaptue, L. (1994). A new subtype of human immunodeficiency virus type 1 (MVP5180) from Cameroon. *J. Virol.* **68,** 1581–1585.

Helseth, E., Kowalski, M., Gabuzda, D., Olshevsky, U., Haseltine, W., and Sodroski, J. (1990). Rapid complementation assays measuring replicative potential of human immunodeficiency virus type 1 envelope glycoprotein mutants. *J. Virol.* **64**(5), 2416–2420.

Hu, D., Dondero, T., Rayfield, M., George, J., Schochetman, G., Jaffe, H., Luo, C., Kalish, M., Weniger, B., Pau, C., Schable, C., and Curran, J. (1996). The emerging genetic diversity of HIV. The importance of global surveillance for diagnostics, research, and prevention. *J. Am. Med. Assoc.* **275,** 210–216.

Kanki, P. J., Hamel, D. J., Sankale, J. L., Hsieh, C., Thior, I., Barin, F., Woodcock, S. A., Gueye-Ndiaye, A., Zhang, E., Montano, M., Siby, T., Marlink, R., NDoye, I., Essex, M. E., and MBoup, S. (1999). Human immunodeficiency virus type 1 subtypes differ in disease progression. *J. Infect. Dis.* **179**(1), 68–73.

Korber, B., Hahn, B., Foley, B., Mellors, J. W., Leitner, T., Myers, G., McCutchan, F., and Kuiken, C. L. (1997) "Human Retroviruses and AIDS 1997: A compilation and analysis of Nucleic Acid and Amino Acid Sequences." Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, NM.

Kostrikis, L., Bagdades, E., Cao, Y., Zhang, L., Dimitriou, D., and Ho, D. (1995). Genetic analysis of human immunodeficiency virus type 1 strains from patients in Cyprus: Identification of a new subtype designated subtype I. *J. Virol.* **69**(10), 6122–6130.

Leitner, T., Escanilla, D., Marquina, S., Wahlberg, J., Brostrom, C., Hansson, H., Uhlen, M., and Albert, J. (1995). Biological and molecular characterization of subtype D, G, and A/D recombinant HIV-1 transmissions in Sweden. *Virology* **209,** 136–146.

Li, Y., Kappes, J., Conway, J., Price, R., Shaw, G., and Hahn, B. (1991). Molecular characterization of human immunodeficiency virus type 1 cloned directly from uncultured human brain tissue: Identification of replication-competent and -defective viral genomes. *J. Virol.* **65**(8), 3973–3985.

Lole, K., Bollinger, R., Paranjape, R., Gadkari, D., Kulkarni, S., Novak, N., Ingersoll, R., Sheppard, H., and Ray, S. (1999). Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. *J. Virol.* **73**(1), 152–160.

Loussert-Ajaka, I., Chaix, M., Korber, B., Letourneur, F., Gomas, E., Allen, E., Ly, T., Brun-Vezinet, F., Simon, F., and Saragosti, S. (1995). Variability of human immunodeficiency virus type 1 group O isolated from Cameroonian patients living in France. *J. Virol.* **69,** 5640–5649.

Mochizuki, N., Otsuka, N., Matsuo, K., Shiino, T., Kojima, A., Kurata, T., Sakai, K., Yamamoto, N., Isomura, S., Dhole, T., Takebe, Y., Matsuda, M., and Tatsumi, M. (1999). An infectious DNA clone of HIV type 1 subtype C. *AIDS Res. Hum. Retroviruses* **15**(14), 1321–1324.

Montano, M., Nixon, C., Ndung'u, T., Bussmann, H., Novitsky, V., Dickman, D., and Essex, M. (2000). Elevated tumor necrosis factor-$\alpha$ activation of human immunodeficiency virus type 1 subtype C in southern Africa is associated with an NF-$\kappa$B enhancer gain-of-function. *J. Infect. Dis.* **181,** 76–81.

Montano, M., Novitsky, V., Blackard, J., Cho, N., Katzenstein, D., and Essex, M. (1997). Divergent transcriptional regulation among expanding human immunodeficiency virus type 1 subtypes. *J. Virol.* **71,** 8657–8665.

Myers, G., Korber, B., Hahn, B., Jeang, K., Mellors, J., McCautchan, F., Henderson, L., and Pavlaskis, G. (1995). "Human Retroviruses and AIDS 1995: A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences." Los Alamos National Laboratory, Los Alamos, NM.

Naghavi, M., Schwartz, S., Sonnerborg, A., and Vahlne, A. (1999). Long terminal repeat promoter/enhancer activity of different subtypes of HIV-1 type 1. *AIDS Res. Hum. Retroviruses* **15,** 1293–1303.

Neilson, J., John, G., Carr, J., Lewis, P., Kreiss, J., Jackson, S., Nduati, R., Mbori-Ngacha, D., Panteleeff, D., Bodrug, S., Giachetti, C., Bott, M., Richardson, B., Bwayo, J., Ndinya-Achola, J., and Overbaugh, J. (1999). Subtypes of human immunodeficiency virus type 1 and disease stage among women in Nairobi, Kenya. *J. Virol.* **73**(5), 4393–4403.

Novitsky, V., Montano, M., McLane, M., Renjifo, B., Vannberg, F., Foley, B., Ndung'u, T., Rahman, M., Makhema, M., Marlink, R., and Essex, M. (1999). Molecular cloning and phylogenetic analysis of human immunodeficiency virus type 1 subtype C: A set of 23 full-length clones from Botswana. *J. Virol.* **73**(5), 4427–4432.

Page, R. D. (1996). TreeView: An application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* **12**(4), 357–358.

Patterson, B. K., Landay, A., Andersson, J., Brown, C., Behbahani, H., Jiyamapa, D., Burki, Z., Stanislawski, D., Czerniewski, M. A., and Garcia, P. (1998). Repertoire of chemokine receptor expression in the female genital tract: Implications for human immunodeficiency virus transmission. *Am. J. Pathol.* **153**(2), 481–490.

Perriere, G., and Gouy, M. (1996). WWW-query: An on-line retrieval system for biological sequence banks. *Biochimie* **78**(5), 364–369.

Ping, L., Nelson, J., Hoffman, I., Schock, J., Lamers, S., Goodman, M., Vernazza, P., Kazembe, P., Maida, M., Zimba, D., Goodenow, M., Eron, J., Jr., Fiscus, S., Cohen, M., and Swanstrom, R. (1999). Characterization of V3 sequence heterogeneity in subtype C human immunodeficiency virus type 1 isolates from Malawi: Underrepresentation of X4 variants. *J. Virol.* **73**(8), 6271–6281.

Preston, B., Poiesz, B., and Loeb, L. (1988). Fidelity of HIV-1 reverse transcriptase. *Science* **242**(4882), 1168–1171.

Proutski, V., and Holmes, E. (1998). SWAN: Sliding window analysis of nucleotide sequence variability. *Bioinformatics* **14**(5), 467–468.

Renjifo, B., Chaplin, B., Mwakagile, D., Shah, P., Vannberg, F., Msamanga, G., Hunter, D., Fawzi, W., and Essex, M. (1998). Epidemic expansion of HIV type 1 subtype C and recombinant genotypes in Tanzania. *AIDS Res. Hum. Retroviruses* **14**(7), 635–637.

Renjifo, B., Gilbert, P., Chaplin, B., Vannberg, F., Mwakagile, D., Msamanga, G., Hunter, D., Fawzi, W., and Essex, M. (1999). Emerging recombinant human immunodeficiency viruses: Uneven representation of the envelope V3 region. *AIDS* **13**(13), 1613–1621.

Sadaie, M., Kalyanaraman, V., Mukopadhayaya, R., Tschachler, E., Gallo, R., and Wong-Staal, F. (1992). Biological characterization of noninfectious HIV-1 particles lacking the envelope protein. *Virology* **187,** 604–611.

Salminen, M. O., Johansson, B., Sonnerborg, A., Ayehunie, S., Gotte, D., Leinikki, P., Burke, D. S., and McCutchan, F. E. (1996). Full-length sequence of an Ethiopian human immunodeficiency type 1 (HIV-1)

isolate of genetic subtype C. *AIDS Res. Hum. Retroviruses* **12**(14), 1329–1339.

Sanchez, G., Xu, X., Chermann, J., and Hirsch, I. (1997). Accumulation of defective viral genomes in peripheral blood mononuclear cells of human immunodeficiency virus type 1-infected individuals. *J. Virol.* **71**(3), 2233–2240.

Siepel, A. C., Halpern, A. L., Macken, C., and Korber, B. T. (1995). A computer program designed to screen rapidly for HIV type 1 intersubtype recombinant sequences. *AIDS Res. Hum. Retroviruses* **11**(11), 1413–1416.

Simon, F., Mauclere, P., Roques, P., Loussert-Ajaka, I., Muller-Trutwin, M., Saragosti, S., Georges-Courbot, M., Barre-Sinoussi, F., and Brun-Vezinet, F. (1998). Identification of a new human immunodeficiency virus type 1 distinct from group M and O. *Nat. Med.* **4,** 1032–1037.

Sullivan, N., Sun, Y., Li, J., Hofmann, W., and Sodroski, J. (1995). Replicative function and neutralization sensitivity of envelope glycoproteins from primary and T-cell line-passaged human immunodeficiency virus type 1 isolates. *J. Virol.* **69**(7), 4413–4422.

Syu, W., Lee, W., Du, B., Yu, Q., Essex, M., and Lee, T. (1991). Role of conserved gp41 cysteine residues in the processing of human immunodeficiency virus envelope precursor and viral infectivity. *J. Virol.* **65**(11), 6349–6352.

Trujillo, J., Wang, W., Lee, T., and Essex, M. (1996). Identification of the envelope V3 loop as a determinant of a CD4-negative neuronal cell tropism for HIV-1. *Virology* **217,** 613–617.

Tscherning, C., Alaeus, A., Fredriksson, R., Bjorndal, A., Deng, H., Littman, D., Fenyo, E., and Albert, J. (1998). Differences in chemokine receptor usage between genetic subtypes of HIV-1. *Virology* **241,** 181–188.

Vanden Haesevelde, M., Decourt, J., De Leys, R., van der Groen, G., van Heuverswijn, H., and Saman, E. (1994). Genomic cloning and complete sequence analysis of a highly divergent African human immunodeficiency virus isolate. *J. Virol.* **68,** 1586–1596.

Wang, W., Dudek, T., Zhao, Y., Brumblay, H., Essex, M., and Lee, T. (1998). CCR5 coreceptor utilization involves a highly conserved arginine residue of HIV type 1 gp120. *Proc. Natl. Acad. Sci. USA* **95**(10), 5740–5745.